Targeted rescue of a destabilized mutant of p53 by an in silico screened drug

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Contributed by Alan R. Fersht, University of Cambridge, Cambridge, United Kingdom, June 1, 2008 (sent for review May 8, 2008)

The tumor suppressor p53 is mutationally inactivated in ~50% of human cancers. Approximately one-third of the mutations lower the melting temperature of the protein, leading to its rapid denaturation. Small molecules that bind to those mutants and stabilize them could be effective anticancer drugs. The mutation Y220C, which occurs in ~75,000 new cancer cases per annum, creates a surface cavity that destabilizes the protein by 4 kcal/mol, at a site that is not functional. We have designed a series of binding molecules from an in silico analysis of the crystal structure using virtual screening and rational drug design. One of them, a carbazole derivative (PhiKan083), binds to the cavity with a dissociation constant of ~150 μM. It raises the melting temperature of the mutant and slows down its rate of denaturation. We have solved the crystal structure of the protein–PhiKan083 complex at 1.5-A resolution. The structure implicates key interactions between the protein and ligand and conformational changes that occur on binding, which will provide a basis for lead optimization. The Y220C mutant is an excellent “druggable” target for developing and testing novel anticancer drugs based on protein stabilization. We point out some general principles in relationships between binding constants, raising of melting temperatures, and increase of protein half-lives by stabilizing ligands.

NMR screen | oncogenic mutant | protein stabilization | virtual drug design | crystal structure

The tumor suppressor p53 is a key protein in the cell’s defense against cancer. If p53 and its associated cell-cycle pathways are active, then p53 will arrest the cell cycle of a potentially cancerous cell and induce apoptosis (1, 2). It is such a potent tumor suppressor that it or its pathways must be inactivated by mutation for cancer to proceed. p53 is inactivated directly by mutation in ~50% of human cancers. It has a complex structure, being composed of several domains (reviewed in ref. 3). Nearly all of the oncogenic mutations occur in its core or DNA-binding domain, contained within the sequence of residues 94-292 (4, 5). The core domain of wild-type protein is rather unstable, with a melting temperature of ~44°C and a short half-life of ~9 min at body temperature (6–8). Many of the oncogenic mutants are inactivated simply because their stability is lowered so that the protein denatures very rapidly and is either too unstable to function at body temperature or rapidly depleted by denaturation and aggregation (9).

In principle, destabilized mutants of p53 can be stabilized by the binding of other molecules, as shown by the binding of a specific double-stranded DNA (6), heparin (10), or a designed peptide (11). Those molecules are targeted against wild-type p53 core domain and should bind generically to most mutants. Other molecules have been proposed to stabilize the folded state of the core domain of p53 [e.g., CP-31398 (12)], but, unlike the aforementioned examples, do not bind reversibly to the core domain (10, 13, 14) and promote anticancer effects in vivo via routes other than thermal stabilization of p53 (15).

We have proposed an alternative strategy to stabilizing p53 in a generic mode: the targeting of specific lesions in the protein that are induced by mutation so a drug may be designed that binds tightly to a mutation-induced binding site but weakly to wild-type p53 (5, 16). Y220C is the ninth most frequent p53 cancer mutant and accounts for an estimated 75,000 new cancer cases per annum worldwide based on cancer incidence statistics by the World Health Organization and reported mutation frequencies (www-p53.iarc.fr) (17). The mutation creates a surface crevice, and the protein is highly destabilized as a result (16). Importantly, this crevice is distant from the surface regions that are known to be involved in DNA recognition or protein–protein interactions, making it a particularly attractive target site for stabilizing small-molecule drugs. Here, we have: discovered a family of molecules that bind to the cavity of the oncogenic mutant Y220C using in silico screening based on the crystal structure and NMR screening within selected compounds; measured the binding of a representative compound, PhiKan083, to the target cavity using NMR spectroscopy; confirmed the dissociation constant by various biophysical methods; and shown that it raises the apparent melting temperature of the Y220C mutant and slows down the rate of thermal denaturation. We have solved the crystal structure of PhiKan083 bound to the mutant Y220C, thus identifying the residues of the protein that are in contact with the small molecule, which will provide a template for the design of drugs that may be therapeutically important.

Results and Discussion

Experiments were performed on the core domain T-p53C-Y220C (16), which has the mutation Y220C in a stabilized framework of human p53 that contains four mutations (18, 19).

In Silico Screening. We applied a structure-based in silico screening approach to the crystal structure of T-p53C-Y220C [Protein Data Bank (PDB) ID code 2JIX] (16). Starting from the ZINC database (release 5) (20), a virtual collection of commercially available screening compounds, we chose a subset of 2,066,906 compounds according to the Lipinski “rule-of-five” (21). We explicitly considered multiple tautomers and protonation states when we created our initial database of 2,529,908 structures. Adding up to 10 alternative low-energy conformations per structure by the high-throughput conformational sampling module of MOE (22), we generated a total pool of >24.8 million conformers. We applied a series of increasingly more sophisticated filters including: (i) structure-based pharmacophore mod...
els [supporting information (SI) Fig. S1] in MOE (22), (ii) ligand docking into the crystal structure with GOLD (23, 24) and rescoring of the best poses to form a consensus score (Fig. S2), and (iii) manual selection from the consensus hit lists according to criteria of medicinal chemistry and crystallography (Fig. 1).

**Primary in Vitro Screening Using 1H/15N-HSQC NMR Spectroscopy.** We screened 80 compounds from the final list of selected molecules in cocktails of four ligands using 1H/15N-HSQC (HSQC, heteronuclear single-quantum coherence) NMR spectroscopy. Four compounds each at 2.5 mM concentration in d6-DMSO were added to 15N-labeled T-p53C-Y220C to give a final concentration of 70 μM of protein, 114 μM of each compound, and 4.6% d6-DMSO. The compounds from any pot that caused significant changes in chemical shifts were then reexamined in a concentration-dependent manner. This increased to 15.7 min at saturating concentrations of PhiKan059 and PhiKan083, respectively, at 10°C (data not shown). Thermal titration of PhiKan083 with T-p53C-Y220C gave 1:1 stoichiometry and a Kd of 125 ± 10 μM (Fig. 4).

**Thermal Stabilization and Kinetics of Denaturation.** We found initially from differential scanning calorimetry that PhiKan059 stabilized T-p53C-Y220C in a concentration-dependent manner. T-p53C-Y220C denatures irreversibly, and its apparent Tm varies with heating rate as does the denaturation of any protein where reversible and irreversible denaturation compete. At very fast heating, the measured Tm approximates to its true value, because the irreversible process is slower than equilibration, which in turn is still fast compared with the heating rate. The Tm is raised nearly 2°C from 316 K by 2.5 mM PhiKan083, and the data fit the equation expected for stabilization by simple binding with an approximate Kd of 140 ± 73 μM at 316–318 K (Fig. 3B).

The kinetics of denaturation of T-p53C-Y220C at 310 K (37°C) was fitted to a simple binding model for PhiKan083 (Fig. 3C). In the absence of ligand, the protein had a half-life of 3.8 min. This increased to 15.7 min at saturating concentrations of PhiKan083.

**Crystal Structure of PhiKan083 Bound to T-p53C-Y220C and Implications for Drug Design.** We solved the crystal structure of the T-p53C-Y220C:PhiKan083 complex at 1.5-Å resolution by soaking the small molecule into crystals of the mutant (Fig. 5A, Table 1). One of the two molecules in the asymmetric unit (chain B) showed unambiguous electron density for a PhiKan083 molecule bound to the mutation-induced surface cleft (Fig. 5C), whereas the pocket was partly occupied in chain A (Fig. S4). The central carbazole moiety is largely buried in the cleft, with the 9-ethyl group occupying the deepest part of the hydrophobic pocket (Fig. 5B). Binding would appear to have an important contribution from hydrophobic packing interactions. The ethyl group is in close contact to the sulfhydryl group of the mutated residue Cys-220, which adopts two alternative conformations, and a number of hydrophobic side chains (Phe-109, Leu-145, Val-147, and Leu-257), thus anchoring the ligand to the pocket. The planar carbazole ring system is sandwiched between the hydrophobic side chains of Pro-222 and Pro-223 on one side, and Val-147 and Pro-151 on the other side of the binding cleft. The ring nitrogen sits close to the position of the hydroxyl group of the tyrosine residue in the wild-type structure (1.0-Å distance) (Fig. 5E). The N-methylmethanamine moiety forms a hydrogen bond with the main-chain carbonyl of Asp-228 (2.7-Å distance). Only very small structural shifts occur upon ligand binding to the mutant. The residues that are within 5 Å of PhiKan083 (residues 109, 145–147, 150, 151, 220–223, 228–230, and 257) superimpose with a rmsd of 0.3 Å (all atoms). The most significant shift is observed for the side chain of Thr-150, which is displaced by up to 1.4 Å upon binding, thus widening the entrance of the pocket (Fig. 5D).

The occupancy of PhiKan083 in the binding pocket upon soaking is significantly different in the two molecules in the asymmetric unit, even though the architecture of the crystal lattice would suggest a similar accessibility of both sites.
Isothermal titration calorimetry of PhiKan083 binding at 20°C shows which may hamper effective binding to molecule A upon soaking alternative conformations in molecule A (but not in molecule B), side chain of Thr-230 inside the binding pocket adopts two ligand-free structure of the mutant (PDB ID code 2J1X), the efficient binding may be reduced in chain A. Moreover, in the packing arrangement, the structural plasticity required for efficient binding is not engaged in packing interactions in molecule B. In this packing arrangement, the structural plasticity required for efficient binding may be reduced in chain A. Moreover, in the ligand-free structure of the mutant (PDB ID code 2J1X), the side chain of Thr-230 inside the binding pocket adopts two alternative conformations in molecule A (but not in molecule B), which may hamper effective binding to molecule A upon soaking of the crystals, because one of these conformations narrows the bottom of the binding pocket (Fig. 5F).

Comparison of modeled and observed binding modes nicely shows how relatively small changes in the protein environment that occur upon ligand binding can be crucial for the accuracy of binding-mode predictions. If the ligand is docked to chain A of the free structure with the Cγ-methyl group of the Thr-230 side chain occupying part of the binding site (purple structure in Fig. 5F), the anchoring ethyl group is positioned at ~1.5 Å from its actual binding position (rmsd of the whole ligand, 6.0 Å), and the carbazole is flipped by 180°, placing the amine group at the opposite end of the binding pocket. Furthermore, this altered binding mode corresponds to a dramatic decrease in predicted affinity, as represented by its DrugScore3D value (26) (Table 2). In contrast, if the ligand is docked into the structure of the complex (after removing the coordinates of the bound ligand), i.e., taking the observed small induced-fit movements of the protein residues into account, the modeled binding mode (yellow molecule in Fig. 5F) almost perfectly matches the observed binding mode (green molecule in Fig. 5F). Small induced-fit structural changes bedevil in silico screening methods, and so it is important to have solved the structure of the complex. Even small changes in the structures of substrates and ligands have been known from early studies to cause radical changes in modes of binding (27).

Inspection reveals contacts, where the central, hydrophobic carbazole scaffold contacts a polar atom. The hydroxyl of Thr-150 at the entrance of the cleft, for example, is in close contact to C5 of the bound carbazole (3.5 Å). Probably more crucially, the main-chain oxygen of Leu-145 at the bottom of the cleft is only 3.3 Å away from C1 of the carbazole ring system. In the unligated structure (PDB ID code 2J1X), this carbonyl group forms a hydrogen bond with a water molecule that is displaced upon ligand binding (Fig. 5D). These observations indicate that a substantial increase in binding energy, and hence a lower dissociation constant, should be achieved through structure-guided modification of the central scaffold, e.g., by providing a hydrogen-bond donor for the carbonyl of Leu-145 or by replacing part of the aromatic structure by suitable nonaromatic bioisosteres (28–30). Moreover, the part of the crevice that preexists in the wild type is not addressed by the binding mode of PhiKan083, which offers the potential for substantial extension of the ligand to create additional stabilizing interactions. Further hit optimi-
Fig. 5. Crystal structure of T-p53C-Y220C in complex with PhiKan083. (A) Ribbon representation of the overall structure of T-p53C-Y220C in complex with PhiKan083 (PDB ID code 2VUK, chain B). PhiKan083 is shown in green as a stick model with its molecular surface. It binds to the mutation-induced cleft on the protein surface that is distant from the known functional interfaces of the protein. The side chain of Cys-220 at the mutation site, which adopts two alternative conformations, is highlighted in orange. (B) Stereoview of the PhiKan083-binding site. p53 residues within a 5-Å distance of the ligand are shown as gray stick models. The protein surface is highlighted in semitransparent gray. (C) \( F_o - F_c \) simulated-annealing omit map of PhiKan083 bound to chain B of T-p53C-Y220C contoured at 3.0σ. (D) Superposition of T-p53C-Y220C in its free (PDB ID code 2J1X chain B; green) and PhiKan083-bound form (yellow), indicating small structural shifts upon ligand binding. PhiKan083 is depicted as a gray stick model. The small red spheres represent water molecules in the ligand-free structure that are displaced upon ligand binding. (E) In wild-type p53, Tyr-220 blocks part of the Phikan083-binding pocket, as shown for the structure of wild-type core domain (PDB code 2AC0, chain B; cyan) (42) superimposed onto PhiKan083-bound T-p53C-Y220C (yellow protein chain and gray PhiKan083 molecule) and free T-p53C-Y220C (green). (F) Docking of PhiKan083 to the structure of ligand-free T-p53C-Y220C (PDB ID code 2J1X, chain A, Thr-230 rotamer A; purple) and to the protein chain of the complex structure (yellow) compared with its actual binding mode in the crystal structure of the complex (green). All images were prepared with PYMOL (http://pymol.sourceforge.net).
Table 1. Data collection and refinement statistics

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**Refinement**

Number of atoms

- Protein: 3,119
- Water: 434
- Zinc: 2
- PhiKan083: 18
- Rsₚ: 16.6
- Rsₚ/rfree: 20.8
- rmsd bonds: 0.009
- rmsd angles: 1.5
- Mean B value, Å²: 15.9

Ramachandran plot statistics

- Most favored/additional allowed, %: 91.0/9.0
- Generously allowed/disallowed, %: 0/0

*Values in parentheses are for the highest-resolution shell.

1$R_{merge} = \sum(h_i-h_o)^2/\sum h_o^2$.
2Number includes alternative conformations.
3$R_{rel} = \sum|F_{o}-|F_{c}|/\sum|F_{o}|$, where $R_{rel}$ was calculated over 5% of the amplitudes chosen at random and not used in the refinement.
4*Calculated with PROCHECK (43).

**Conclusions**

We have demonstrated that the site of mutation in the oncogenic Y220C mutant is a druggable target. PhiKan083 binds to it with reasonable affinity. The crystal structure of the complex will be a starting point for further rounds of drug design and refinement. Because the site of mutation does not appear to be in a region of the protein that is functionally important, it is an excellent target for drug stabilization therapy. The degree of stabilization provided by a drug is related to:

$$T - T_m(1 - (R/\Delta S_{DN(Tm)})\ln(1 + [L]/K_d)),$$

where $T$ is the observed melting temperature, $T_m$ that in the absence of ligand (drug), $K_d$ its dissociation constant, and $\Delta S_{DN(Tm)}$ the entropy of denaturation at $T_m$ (see legend to Fig. S5). For $\Delta S_{DN(Tm)}$ for a protein such as p53, increases in $T_m$ of 3.7, 4.8, 6.2 and 7.3°C are expected for the ratios of $[L]/[K_d]$ of 10, 20, 50, and 100 respectively; see Fig. S5. Further, the half-life of the mutant protein is significantly increased by the drug, and the increase will be greater for future generations of drugs that have more intrinsic binding energy. There is every possibility that an anticancer drug could be developed for the Y220C mutant using compounds that bind more tightly than the current lead.

**Methods**

**Materials.** T-p53C-Y220C for protein crystallography was purified as described (16, 33). For biophysical measurements and NMR spectroscopy, we used a pET24a-HLT-derived vector (courtesy of M. D. Allen, Medical Research Council Centre), containing an N-terminal fusion of a 6xHis/lipoamyl domain/TEV protease cleavage site sequence. Expression and purification followed published protocols (34). The N-terminally fused 6xHis/lipoamyl domain sequence was removed by TEV protease digestion. PhiKan059 and PhiKan083 were obtained from Enamine, Ltd.

**Biophysical Methods. Isothermal titration calorimetry.** Binding was measured with an isothermal titration calorimeter (VP-ITC, MicroCal) at 20°C in a 25 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, and 1 mM DTT. For binding of PhiKan083 to T-p53C-Y220C, 5 mM PhiKan083 in 5% d6-DMSO in buffer was titrated into the sample cell containing 100 µM protein in 5% d6-DMSO in buffer. The DMSO content was matched using a high-precision Mettler Toledo balance. Injection steps were 10 µl (first injection, 3 µl) with 600-s spacing. Further data evaluation was done by using the MicroCal Origin program.

**NMR spectroscopy.** The low-molecular-weight compounds were dissolved in d6-DMSO to make 10 mM stock solutions. To screen compound mixtures by chemical-shift mapping, 10 µl each of four different compounds were mixed together, and 25 µl of this mixture was added to 25 µl of D₂O and 500 µl of 70 µM T-p53C-Y220C (in 25 mM sodium phosphate, 150 mM NaCl, and 5 mM DTT, pH 7.2). The final concentration for each compound was 114 µM at a concentration of 4.5% (vol/vol) d6-DMSO. NMR samples were freshly prepared and kept sealed under argon after degassing. H15N HSQC correlation spectra were acquired at 20°C on Bruker Avance + 700 and Avance 800 spectrometers using a H15N/C15N triple resonance inverse, cysgenic 5-mm probe (Bruker), with the following parameters: 16 scans, 128 complex points in t1, recycle time of 0.95 sec, and 1,024 total points in t2. Using Bruker’s TopSpin 2.0 software, the number of complex points in t1 was doubled by forward-complex linear prediction, and shifted squared sine bell window functions were applied to both dimensions before zero filling and Fourier transformation. A 2D resolution of 2.0 Hz per point in the H15N frequency dimension and 4.7 Hz per point in the 15N frequency dimension was used. Spectra were analyzed by using Sparky 3.113 (36).

**Thermal stability.** Thermal unfolding was followed either by differential scanning calorimetry as described in ref. 37 at a scan rate of 250 K/h or by monitoring unfolding by the binding of the dye SYPRO Orange (5–x) using a Rotor-gene 6000 (Corbett Life Science) at 270 K/h in 25 mM sodium phosphate, 150 mM NaCl, and 5 mM DTT, pH 7.2, with a protein concentration of 10 µM. Time-dependent fluorescence studies. Unfolding kinetics was performed as described by Friedler et al. (11) at 37°C in 50 mM Heps, pH 7.2, 1 mM Tris-2-carboxyethylphosphine, by following the emission of tryptophan at 340 nm on excitation at 280 nm (6), using a Cary Eclipse fluorescence spectrophotometer controlled by the supplied Cary software. Reactions were followed for 10,000 s. Data were fitted to a single exponential followed by a linear-drift term.

**X-Ray Crystallography Methods.** Crystals of T-p53C-Y220C in space group P2₁2₁2₁, with two molecules in the asymmetric unit were grown as described (16, 33). For biophysical measurements and NMR spectroscopy, we used a pET24a-HLT-derived vector (courtesy of M. D. Allen, Medical Research Council Centre), containing an N-terminal fusion of a 6xHis/lipoamyl domain/TEV protease cleavage site sequence. Expression and purification followed published protocols (34). The N-terminally fused 6xHis/lipoamyl domain sequence was removed by TEV protease digestion. PhiKan059 and PhiKan083 were obtained from Enamine, Ltd.
significant difference density having contributions from PhiKan083 in the same binding mode as in chain B but bound with a low occupancy and a network of water molecules as observed in the unbound state (Fig. S4), but we did not include the ligand in the final model of chain A. The data collection and refinement statistics are shown in Table 1.

ACKNOWLEDGMENTS. We thank Caroline Blair for protein purification for crystalllographic studies. Dr. Chris Johnson for advice and helpful discussions. Dr. Chris Johnson for advice and helpful discussions. Dr. Chris Johnson for advice and helpful discussions.
Pharmacophore Model:

11 donor positions
8 acceptor positions
6 donor-acceptor positions
7 donor peer positions
5 acceptor peer positions
1 donor peer-acceptor peer position
5 aromatic-hydrophobic positions
2 aromatic axis positions

45 pharmacophore features
479 spheres defining the protein excluded volume

constraints:
2 essential Aro-Hyd
> 7 features partial match

Fig. S1. Pharmacophore model used for the virtual screening procedure. Features are color-coded and explained.
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<td>-0.020</td>
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<td>0.733</td>
<td>0.018</td>
<td>-0.046</td>
<td>0.072</td>
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Fig. S2. Similarity and complementarity of different scoring functions. The correlation coefficient of pairs of scoring functions over all 13,145 molecules is given as numbers and used for color-coding the table. High numbers close to 1 therefore indicate high correlation and redundancy between the scoring functions, low numbers close to 0 indicate less correlation and thus more complementarity.
Fig. S3. $^1$H/$^{15}$N-HSQC spectrum of 70 μM T-p53C-Y220C in the presence of various concentrations of PhiKan083 (red: 0 μM; orange: 68 μM; yellow: 114 μM; green: 227 μM; cyan: 341 μM; blue: 455 μM; purple: 909 μM). The lower four panels show close-up views of the spectrum for selected residues with significant changes in chemical shifts upon addition of the ligand.
Fig. S4. Differential binding efficiency of Phikan083 to the two molecules in the asymmetric unit of T-pS3C-Y220C crystals upon soaking. (A) $|F_o - F_c|$ electron density map at a contour level of 2.5 $\sigma$ next to Cys-220 in chain A of the structure of T-pS3C-Y220C (orange) in complex with Phikan083, indicating partial occupancy of the binding pocket by Phikan083, with a binding mode similar to that observed in chain B (green structure). Selected water molecules of chain A in the ligand-free structure (PDB ID code 2J1X, magenta) are shown as red spheres. (B) $|F_o - F_c|$ simulated annealing omit map of Phikan083 bound to chain B of T-pS3C-Y220C contoured at 3.0 $\sigma$. Residues 221–223 and 257 of the binding pocket are omitted for clarity in both panels. The figure was prepared using PYMOL (http://pymol.sourceforge.net)
Fig. S5. Increase in $T_m$ of a protein on addition of ligand. In absence of ligand (L), suppose the protein melts at a temperature $T_m$. In the presence of [L], the protein appears to melt at temperature $T$, where the concentration of D, the denatured state, is 50% of the total protein and the sum of the concentrations N (Native state) and NL complex constitute the other 50%. Suppose D is in equilibrium with N with equilibrium constant $K_{N/D} = [N]/[D]$, and $K_d = [N][L]/[NL]$. Then, at T, $[D] = [N] + [NL] = [N](1 + [L]/K_d)$. That is, $[D]/[N] = (1 + [L]/K_d)$. Also, $[D]/[N] = \exp(-\Delta G_{D-N}(T)/RT)$, where $\Delta G_{D-N}$ is the free energy of denaturation at T. Therefore, $\Delta G_{D-N} = -RT \ln(1 + [L]/K_d)$. We can relate the observed changes in T to $\Delta G_{D-N}$ using the equation $d\Delta G_{D-N}/dT = -\Delta S_{D-N}$. For small changes in temperature, $\Delta G_{D-N}(T) = -\Delta S_{D-N}(T-m)$. So that, $\Delta S_{D-N}(T)/RT = -\ln(1 + [L]/K_d)$, which rearranges to: $T = T_m/(1 - (R/\Delta S_{D-N}(T))\ln(1 + [L]/K_d))$. (To a further approximation, $\Delta T = T_m (R/\Delta S)\ln(1 + [L]/K_d))$. Plotted is the increase in $T_m$ for a protein where $\Delta S = 400 \text{ cal/mol/K}$, the approximate value for the core domain of p53.